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Diversity of Tn*1546* Elements in Clinical Isolates of Glycopeptide-Resistant Enterococci from Scottish Hospitals

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The Tn*1546*-related elements of 48 Van glycopeptide-resistant enterococci were compared. Ten distinct Tn*1546* types were identified with variation primarily due to IS*1542* and IS*1216V*-like insertions. Clonal isolates frequently differed in their Tn*1546* type, indicating instability of Tn*1546*-related elements. A putative hybrid promoter was identified, generated upstream of *vanR* by the insertion of IS*1542*. The presence of this hybrid promoter was associated with constitutive expression of the *van* genes and elevated teicoplanin resistance.

While all VanA phenotype glycopeptide-resistant enterococci (GRE) share the same basic Tn*1546* structure, as described for *Enterococcus faecium* BM4147 (2), considerable diversity has now been identified within Tn*1546*-related elements. This variation, in the form of point mutations, insertion sequence (IS) elements, and deletions, has been exploited in several epidemiological studies (8, 10, 13), often in combination with well-established methods (e.g., pulsed-field gel electrophoresis [PFGE] and ribotyping). However, the possible transient nature of insertion sequences has led to questions about the suitability of this type of analysis in epidemiological studies (4, 15). In this study, we have compared 48 VanA enterococcal isolates by PFGE and by molecular analysis of Tn*1546*-related elements.

Forty-eight clinical isolates of enterococci, collected from eight hospitals in Scotland over a 5-year period (1995 to 1999) were confirmed as *vanA* positive by PCR (5) and identified with the AP120 Strep system (BioMerieux). Sixty-nine percent of isolates were *E. faecium*, and the remainder were *E. faecalis*. Vancomycin and teicoplanin MICs were determined by incorporation of the antimicrobial agents into Mueller-Hinton agar (Oxoid) and inoculation of plates with approximately 10^4 CFU per inocula. All 48 isolates displayed resistance levels that were typical of the VanA phenotype. Vancomycin and teicoplanin MICs ranged from 64 to 1,024 mg/liter and 8–128 mg/liter, respectively.

PFGE analysis and interpretation were performed as previously described (9, 12). Following digestion with *Sma*I, genomic DNA was separated by electrophoresis for 24 h at 200 V with 5- to 40-s pulse times. Discounting those isolates belonging to a previously described outbreak strain of VanA *E. faecium* (3), the 48 isolates were largely heterogeneous in nature, with only small discrete clusters of related isolates identified. No GRE isolated from different geographic regions of Scotland shared PFGE patterns.

Template DNA for PCRs was prepared by the guanidium thiocyanate extraction method (11). Long-template PCR (L-PCR; Expand long-template PCR system; Boehringer Mannheim) was used according to the manufacturer's instructions. The inverted repeat (IR)-specific primers described previously (16) enabled amplification of the Tn*1546*-related elements and subsequent restriction fragment length polymorphism (RFLP) analysis by *Cla*I digestion. Five of the 48 isolates failed to yield an L-PCR product with the Tn*1546*-IR primer, suggesting that they lacked at least one of the IRs. The loss of the left IR has been previously described and is often associated with the presence of IS*1216V*-like elements alone or in combination with a truncated IS*3*-like element (6, 13). The reason for the L-PCR failure in these five isolates was not ascertained. The Tn*1546*-related elements of the remaining 43 isolates were assigned to 10 distinct types on the basis of *Cla*I RFLP analysis. Fourteen isolates harbored Tn*1546* elements that were indistinguishable from the prototype Tn*1546* element by *Cla*I RFLP analysis. The remaining 29 isolates harbored nonprototype elements. Different Tn*1546* types were evident in clonally related isolates, indicating instability within Tn*1546*-related elements. Such instability has been previously described (15).

Using the primers listed in Table 1, all nonprototype Tn*1546*-related elements were further studied by PCR and by hybridization analysis with the ECL (enhanced chemiluminescence) random prime labeling and detection system (Amersham Life Sciences Ltd.). PCR products of interest were sequenced in both directions by the dideoxy method on an ABI Prism automated sequencer. All 29 nonprototype elements harbored IS*1542* within the *orf2-vanR* intergenic region (nucleotide position 3932) and an IS*1216V*-like element within the *vanX-vanY* intergenic region (nucleotide position 8839). Both IS elements have been described previously at the same nucleotide positions (13, 15). In addition, all nonprototype Tn*1546*-related elements had considerable variation within the *orf1-orf2* region. Hybridization analysis, performed following *Bam*HI digestion of Tn*1546*-related elements, confirmed that this variation was due at least in part to the insertion of an IS*1216V*-like element within the *orf1-orf2* region. The precise location of the insertion was not ascertained.

The point mutation at Tn*1546* nucleotide position 8234 (G→T) within the *vanX* gene was screened for as described

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TABLE 1. Primers used in the analysis of Tn*1546*-related elements[a]

Primer	Sequence	Coordinates	Reference
Amplification			
Tn*1546*-IR	5′-GGAAAATGCGGATTTACAACGCTAAG	13–38; 10839–10814	16
orf1-f	5′-GATGGTGGCTCCTTTTCC	906–923	
orf1-r	5′-CCATTGTTGGCAGCAGAC	1597–1580	
vanR-f	5′-GCCGAAAAGCCCTTTGATA	3841–3859	
vanR-r	5′-TCGTTCTAGTTTGGAATAGTCG	4699–4678	
vanS-f	5′-TAGGGTAGAGCTTCCAGCGA	5752–5771	
vanH-r	5′-ATTATCGTTGCCATAACGCC	6115–6096	
vanX-f	5′-GATGAACGCTCTCATCATGC	8448–8467	
vanY-r	5′-TTCCTGAGAAAACAGTGCTTCA	9138–9117	
vanX1	5′-ACTTGGGATAATTTCACCGG	8082–8101	8
vanX2	5′-TGCGATTTTGCGCTTCATTG	8505–8486	8
Sequencing			
1216V-A	5′-TTGAAGATGTAAGGCAGAGC	8727–8746	
1216V-B	5′-AGGCTGAACTGCCTGTTGAC	8857–8838	
1216V-C	5′-GATCGCATAGAGGGGTGGTA	8802–8821	
1542-A	5′-TAGCTGTTCGAGCGAGTTCA	980–961[b]	
1542-B	5′-TCGGCAATTTCATGTTCATC	4022–4003	
Hybridization			
1216V-D	5′-GTACAGACCGAAAACCCGAA	88–107[c]	
1216V-E	5′-GCAATTTCAGCAGGATGTGA	714–695[c]	

[a] Coordinates are based on the published Tn*1546* sequence (GenBank accession number M97297) unless stated otherwise. Sequencing primers were used in the sequencing of the IS*1542* and IS*1216V*-like elements within the *orf2-vanR* and *vanX-vanY* intergenic regions, respectively. Hybridization primers 1216V-D and 1216V-E were used to obtain IS*1216V*-like-specific PCR product from which labeled probes were produced. Primers without a reference were designed by Primer3 (http://www.genome.wi.mit.edu.cgi-bin/primer).

[b] Corresponds to the published sequence of IS*1542* (GenBank accession number AF114715).

[c] Corresponds to the published sequence of the IS*1216V*-like element (GenBank accession number AF093508).

previously (10). This point mutation, previously associated with porcine isolates of VanA GRE (14), was not evident in any of the isolates studied.

Sequencing of *orf2-vanR* intergenic regions harboring IS*1542* identified a putative hybrid promoter. The -10 TATAAT box that forms part of the native *vanR* promoter proposed by Holman et al. (7) is duplicated by the 8-bp target site duplication generated by IS*1542* insertion at nucleotide position 3932. This duplicated -10 box forms a putative promoter sequence in conjunction with an outwardly directed -35 box (TTTACA) located within the inverted repeat of IS*1542*. The impact of the IS*1542* insertion and the resulting hybrid promoter on the expression of glycopeptide resistance was assessed by growth curve analysis following glycopeptide challenge and by VanX D,D-dipeptidase enzyme assays (1). Growth curves were consistent with induced expression of *van* genes, irrespective of whether encoded by a prototype or nonprototype Tn*1546* element. However, VanX enzyme assays revealed significant constitutive expression of the *van* genes of nonprototype Tn*1546*-related elements. Specific activities in the absence of induction were, on average, 10-fold greater than the background expression from prototype Tn*1546* elements. Constitutive expression, which we propose is mediated by the IS*1542*-generated hybrid promoter, supplemented rather than replaced the inducible *van* gene expression. In addition, nonprototype Tn*1546* elements conferred higher levels of teicoplanin resistance than did prototype Tn*1546* elements. While this phenomenon was partly medium dependent (Table 2), the consistency of teicoplanin MICs conferred by prototype Tn*1546* elements suggested that the elevated resistance was not solely attributable to the media used. The reason for the medium dependency is unclear.

In conclusion, this study has revealed considerable diversity within the Tn*1546*-related elements of VanA GRE in Scotland. The types of variation witnessed were consistent with the findings of previous studies. Many IS elements have the potential to form hybrid promoters, owing to the presence of outwardly directed -35 regions within their inverted repeats. This study describes the first such case within the *van* gene cluster. We propose that constitutive expression of the *van* genes from the

TABLE 2. Summary of teicoplanin MIC data for VanA isolates with prototype and nonprototype Tn*1546* elements

Medium used for sensitivity testing[a]	Prototype Tn*1546*			Nonprototype Tn*1546*[b]		
	MIC_{50}[c]	MIC_{90}[c]	Range[c]	MIC_{50}	MIC_{90}	Range
Iso-Sensitest agar	8	32	4–32	64	128	16–128
Mueller-Hinton agar	16	64	4–64	32	64	16–128
Brain heart infusion agar	16	32	8–32	256	512	32–1024

[a] The control organism, *E. faecalis* NCTC 775, was consistently susceptible on all media tested (MIC, 0.5 mg/liter).

[b] Defined as having IS*1542* and IS*1216V*-like insertions within the *orf2-vanR* and *vcnX-vanY* intergenic regions, respectively.

[c] Expressed in milligrams per liter.

hybrid promoter results in the elevated teicoplanin resistance conferred by nonprototype Tn*1546* elements. The elevation in teicoplanin resistance and not vancomycin resistance could potentially reflect different abilities of the glycopeptide agents to act against residual D-Ala-D-Ala-terminating precursors.

REFERENCES

1. **Arthur, M., F. Depardieu, P. Reynolds, and P. Courvalin.** 1996. Quantitative analysis of the metabolism of soluble cytoplasmic peptidoglycan precursors of glycopeptide-resistant enterococci. Mol. Microbiol. **21:**33–44.
2. **Arthur, M., C. Molinas, F. Depardieu, and P. Courvalin.** 1993. Characterization of Tn*1546*, a Tn*3*-related transposon conferring glycopeptide resistance by synthesis of depsipeptide peptidoglycan precursors in *Enterococcus faecalis* BM4147. J. Bacteriol. **175:**117–127.
3. **Brown, A. R, S. G. B. Amyes, R. Paton, W. D. Plant, G. M. Stevenson, R. J. Winney, and R. S. Miles.** 1998. Epidemiology and control of vancomycin-resistant enterococci (VRE) in a renal unit. J. Hosp. Infect. **40:**115–124.
4. **Darini, A. L. C., M. F. I. Palepou, and N. Woodford.** 2000. Effects of the movement of insertion sequences on the structure of VanA glycopeptide resistance elements in *Enterococcus faecium*. Antimicrob. Agents Chemother. **44:**1362–1364.
5. **Dutka-Malen, S., S. Evers, and P. Courvalin.** 1995. Detection of glycopeptide resistance genotypes and identification to the species level of clinically relevant enterococci by PCR. J. Clin. Microbiol. **33:**24–27.
6. **Handwerger, S., and J. Skoble.** 1995. Identification of chromosomal mobile element conferring high-level vancomycin resistance in *Enterococcus faecium*. Antimicrob. Agents Chemother. **39:**2446–2453.
7. **Holman, T. R., Z. Wu, B. L. Wanner, and C. T. Walsh.** 1994. Identification of the DNA-binding site for the phosphorylated VanR protein required for vancomycin resistance in *Enterococcus faecium*. Biochemistry **33:**4625–4631.
8. **Jensen, L. B., P. Ahrens, L. Dons, R. N. Jones, A. M. Hammerum, and F. M. Aarestrup.** 1998. Molecular analysis of Tn*1546* in *Enterococcus faecium* isolated from animals and humans. J. Clin. Microbiol. **36:**437–442.
9. **Miranda, A. G., K. V. Singh, and B. E. Murray.** 1991. DNA fingerprinting of *Enterococcus faecium* by pulsed-field gel electrophoresis may be a useful epidemiologic tool. J. Clin. Microbiol. **29:**2752–2757.
10. **Palepou, M. F. L., A. M. A. Adebiyi, C. H. Tremlett, L. B. Jensen, and N. Woodford.** 1998. Molecular analysis of diverse elements mediating VanA glycopeptide resistance in enterococci. J. Antimicrob. Chemother. **42:**605–612.
11. **Pitcher, D. G., N. A. Saunders, and R. J. Owen.** 1989. Rapid extraction of bacterial genomic DNA with guanidium thiocyanate. Lett. Appl. Microbiol. **8:**151–156.
12. **Tenover, F. C., R. D. Arbeit, R. V. Goering, P. A. Mickelsen, B. E. Murray, D. H. Persing, and B. Swaminathan.** 1995. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. J. Clin. Microbiol. **33:**2233–2239.
13. **Willems, R. J. L., J. Top, N. van den Braak, A. van Belkum, D. J. Mevius, G. Hendriks, M. Santen-Verheuvel, and J. D. A. van Embden.** 1999. Molecular diversity and evolutionary relationships of Tn*1546*-like elements in enterococci from humans and animals. Antimicrob. Agents Chemother. **43:**483–491.
14. **Woodford, N.** 1998. Glycopeptide-resistant enterococci: a decade of experience. J. Med. Microbiol. **47:**849–862.
15. **Woodford, N., A. M. A. Adebiyi, M. F. I. Palepou, and B. D. Cookson.** 1998. Diversity of VanA glycopeptide resistance elements in enterococci from humans and nonhuman sources. Antimicrob. Agents Chemother. **42:**502–508.
16. **Woodford, N., A. P. Watson, and P. R. Chadwick.** 1997. Investigation by long PCR of the genetic elements mediating VanA glycopeptide resistance in enterococci from uncooked meat in South Manchester, p. 409–412 *In* T. Horaud, A. Bouvet, R. Leclercq, H. de Montclos, and M. Sicard (ed.), Streptococci and the host. Plenum Publishing Corporation, New York, N.Y.